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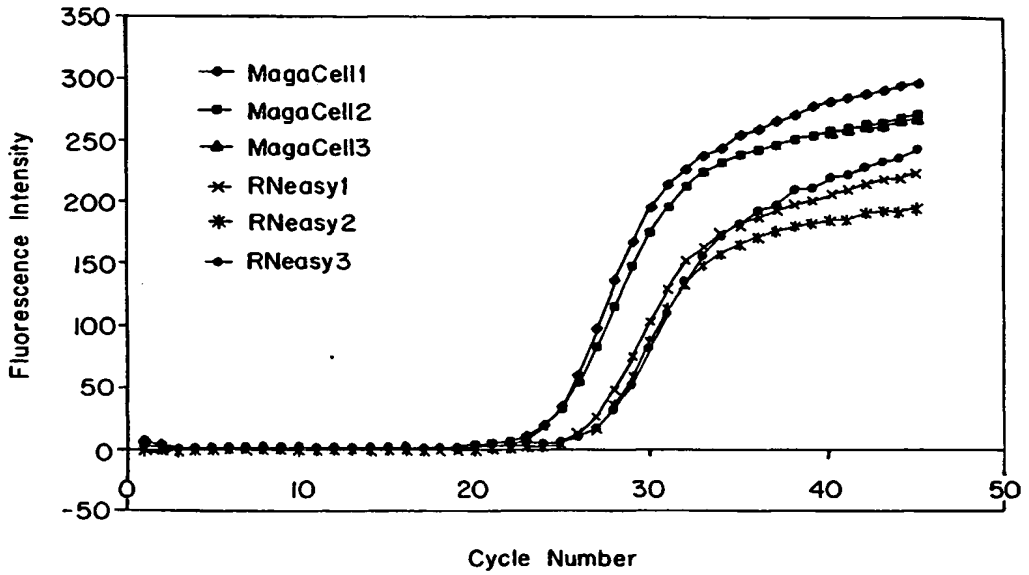
(54) Title: MAGNETIC ISOLATION AND PURIFICATION OF NUCLEIC ACIDS



(57) Abstract: A method for the isolation and purification of nucleic acids such as DNA, RNA, and PNA from various sources using magnetizable cellulose or its derivatives. Adjusting the concentrations of the salt and polyalkylene glycol to the levels that result in binding of nucleic acids to the magnetizable cellulose or its derivatives. Separating the nucleic acids bound to the magnetizable cellulose particles or its derivatives and eluting the nucleic acids from the particles.

WO 02/066993 A1

Magnetic Isolation and Purification of Nucleic Acids

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims the benefit of USSN 60/269,729, filed February 16, 2001, the disclosure of which is incorporated herein by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable

BACKGROUND OF THE INVENTION

Isolation and purification of high quality nucleic acids are critical steps in molecular biology procedures. A number of methods have been reported for the isolation of single and double stranded DNA from biological fluids such as human blood, serum, cultured cells, as well as plants, animal and human tissues, and other specimens. Many different procedures have been described. See, for example, Taylor, J.I., et al., *J. Chromatography A*, 890:159-166 (2000); Ahn, S.C., et al., *BioTechniques*, 29:466-468 (2000); Scott Jr, D.L. et al., *Lett. Appl. Microl.*, 31:95-99 (2000); Lin,Z, and Floros, J., *BioTechniques*, 29:460-466 (2000); Smith, C.E. and York,C.K., U.S. Patent No.6,027,945 (2000); Mrázek, F. and Petrek, M., *Acta Univ. Palacki. Olomuc., Fac. Med.* 142:23-28 (1999); Hawkins, T., U.S. Patent No. 5,898,071 (1999); Hawkins, T., U.S. Patent No.5,705,628 (1998); Davies, M.J., et al., *Anal. Biochem.* 262:92-94 (1998); Levison, P.R., et al., *J. Chromatography A*, 816:107-111 (1998); Rudi, K., et al., *BioTechniques*, 22:506-511 (1997); Kotsopoulos, S.K., and Shuber, A.P., *BioTechniques*, 20:198-200 (1996); Boom, W. R., et al., U.S. Patent No.5,234,809 (1993); Reeve, M.A., WO 91/12079 (1991); Sambrook, J., et al., in: MOLECULAR CLONING, A LABORATORY MANUAL, 2ND EDITION, 1.21-1.45 (1989), Cold Spring Harbor Laboratory Press. Most of these procedures are time consuming, tedious, and costly. In addition a number of these procedures involve the use of hazardous organic solvents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an Agarose gel electrophoresis showing DNA isolated from whole blood using MagaCell™ or Qiagen QIAamp DNA Mini Kit, and shows the high molecular weight non-degraded DNA isolated by both techniques. Lane 1 is a 1 Kb DNA ladder; Lane 2 is calf thymus DNA control; Lanes 3, 5, 7, 9, and 11 are DNA isolated by the present method; and Lanes 4, 6, 8, 10 and 12 are DNA isolated by QIAamp.

Figure 2 is an Agarose gel electrophoresis of plasmid DNA isolated from bacterial cell lysates, using MagaCell™ or Qiagen QIAprep Miniprep Kit, and shows that two different sizes of high quality plasmid DNA were isolated by both techniques. Lanes 1 and 12 are 1 Kb DNA ladders; Lane 2 is plasmid DNA PBA117 control; Lanes 3, 4, 6, and 7 are plasmid DNA PBA117 isolated by MagaCell™; Lanes 5 and 8 are plasmid DNA PBA117 isolated by QIAprep Miniprep; Lanes 9 and 10 are plasmid DNA PBA8 isolated by MagaCell™; and Lane 11 is plasmid DNA PBA8 isolated by QIAprep Miniprep.

Figure 3 is a graph illustrating the real time RT-PCR quantitation of MS2 Viral RNA isolated by MagaCell™ or RNeasy Kit.

DETAILED DESCRIPTION OF THE INVENTION

General

The present method simplifies the isolation of nucleic acids from various sources by eliminating the need for centrifugation or organic solvents including alcohol extraction or washes, and produces nucleic acids ready for further characterization and downstream processing such as PCR, sequencing or blotting procedures. Because of the unique features described herein, the present method is readily adaptable to automation including high throughput screening systems.

Additionally, the iron oxide, cellulose and cellulose derivatives used for the production of magnetizable cellulose in the present invention are commercially available and inexpensive. The method described herein also avoids the lengthy procedure and use of hazardous chemicals involved in the preparation and modification of the magnetic particles described in Hawkins, U.S. Patent No. 5,898,071. Still further, the present methods eliminate the need for chemical synthesis of various functional groups, a requirement for particles

magnetizable cellulose is sufficient to avoid saturation of the cellulose particle surface and at least 60%, more preferably 80% and still more preferably 90% or more of the nucleic acids in a solution are bound to the magnetizable cellulose. In many instances, the portion of nucleic acids bound will be 100%. In some embodiments, however, selective binding of nucleic acids of a particular size can be achieved by manipulation of the salt and polyalkylene glycol concentrations such that only about 5% to about 30% of the total nucleic acid content in a sample is bound to the magnetizable cellulose.

In the methods of the present invention, the magnetizable cellulose can be purchased from Cortex Biochem Inc., San Leandro, CA. Alternatively, the particles can be produced using the procedure described by Pourfarzaneh et al, *Methods Biochem. Anal.* 28, 267-295 (1982). The iron oxide, cellulose and cellulose derivatives used for the production of magnetizable cellulose or magnetizable cellulose derivatives are also commercially available and are inexpensive.

As described in the present invention, the binding of nucleic acids to the magnetizable cellulose or its derivatives and removal of the non-specifically adsorbed proteins or other substances can be achieved using a solution of salt and polyalkylene glycol at certain concentrations. Useful salts in the present invention are selected from LiCl, $BaCl_2$, $MgCl_2$, $CsCl_2$, $CaCl_2$, NaCl, KCl and KI. Preferably the salt is NaCl. Similarly, a variety of polyalkylene glycols are useful in the present invention including, for example, polyethylene glycol and polypropylene glycol. Preferably, the polyalkylene glycol is polyethylene glycol. The salt and polyalkylene reagents are used in concentrations that facilitate binding of nucleic acids to the cellulose coated magnetizable particles and its derivatives. Salt concentrations in the binding and wash buffers will depend on the salt being used and milieu from which the nucleic acids are to be isolated and purified. Generally, the salt concentrations will be about 0.25 M to about 5.0 M. More preferably, the salt concentration in the binding and wash buffers is about 0.5 M to about 2.5 M. Still more preferably, the salt concentration is about 0.5 M to about 1.5 M. Most preferably, the salt concentration of the binding buffer is about 1.25 M and the salt concentration of the wash buffer is about 0.5 M. Similarly, the polyalkylene concentration will depend on the polyalkylene used. Polyethylene glycol is commercially available from suppliers such as Sigma Chemical Company (St. Louis, Missouri, USA) and is useful in molecular weights of about 1,000 to about 10,000, preferably about 6,000 to about 8,000. Depending on the weight range of polyethylene glycol used, the concentration can be adjusted. Generally, for methods in which polyethylene glycol having

particles while leaving the DNA bound to the magnetizable cellulose particles. More preferably, the DNA bound to the magnetizable cellulose particles is eluted with an elution buffer that releases the DNA bound to the magnetizable particles, and the DNA is isolated.

In other preferred embodiments, the nucleic acids in solution are a lysate, preferably prepared from cells of human, plant, animal, viral or bacterial origin. Thus, in one application, the cells are from animals, more preferably humans. In another application, the cells are from plants. In another application, the cells are of bacterial origin. In still another application, the cells are of viral origin.

The nucleic acids that are separated from non-nucleic acid materials (e.g., peptides, proteins, oligosaccharides, lignans, small molecule natural products and other materials typically of natural origin) are generally obtained in a purity of at least 80%, more preferably at least 90%, still more preferably at least 95%, and most preferably at least 99% or more. Accordingly, the present methods are suitable to remove at least 80%, more preferably at least 90%, still more preferably at least 95%, and most preferably at least 99% or more of the non-nucleic acid materials in a particular sample (e.g., a cell lysate).

In yet another aspect of the invention, magnetizable cellulose derivatives are used. Accordingly, the invention provides a method to bind nucleic acids to magnetizable cellulose derivatives comprising:

a) combining magnetizable cellulose derivatives with a solution containing nucleic acids, thereby producing a combination; and

b) adjusting the salt and polyalkylene glycol concentrations of the combination to concentrations suitable for binding the nucleic acids onto the magnetizable cellulose derivatives, whereby all or a portion of the nucleic acids in the solution bind to the magnetizable cellulose derivatives.

Again, the preferred components and amounts are essentially as provided above. The magnetizable cellulose derivatives are, in one group of embodiments, selected from cellulose-CM, cellulose-DEAE and mixtures thereof. Additionally, this method as well as the other methods of the present invention find wide application in the purification of, for example, DNA, RNA, PNA or derivatives thereof.

In related methods, the present invention provides a method of separating nucleic acids from non-nucleic acid materials, comprising:

a) combining magnetizable cellulose derivatives with a solution containing nucleic acids and non-nucleic acid materials to provide a first combination;

1. In a 2 ml microcentifuge tube containing 50 µg (50 µl of a 1 mg/ml DNA solution in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) add 430 µl of the Binding Buffer (10% PEG 8000 MW, 1.25 M NaCl) and 1 mg (20 µl of a 50 mg/ml suspension) of the MagaCell Particles (Cortex Biochem, CA).
2. Mix the tube content at room temperature for 10 minutes, using an end-over-end rotator.
3. Sediment the DNA bound to MagaCell Particles using a magnetic rack.
4. Wash particles with the Wash Buffer (10% PEG 8000 MW, 2.5 M NaCl). Repeat the wash step once more.
5. Elute the DNA from MagaCell Particles using the Elution Buffer (deionized water or TE Buffer [10 mM Tris-HCl, pH 8.0, 1 mM EDTA]).

Agarose gel electrophoresis of the eluted DNA showed a single non-degraded high molecular weight DNA band (Figure 1).

EXAMPLE 2

DNA Isolation Using Magnetizable Cellulose Derivatives

Example 1, described above was repeated using magnetizable cellulose derivatives. These included: MagaCell™-CM and MagaCell™-DEAE (both obtained from Cortex Biochem, San Leandro, CA).

Results obtained with the MagaCell™ derivatives were comparable to those obtained by MagaCell™.

EXAMPLE 3

DNA Isolation from Whole Blood Using Magnetizable Cellulose

DNA from human whole blood samples was released using proteinase K and a specially formulated lysis buffer. The DNA was then bound to MagaCell Particles in presence of the Binding Buffer. The DNA bound to MagaCell Particles was then separated and washed from other contaminants. The DNA was eluted from the particles. The following procedure was used:

1. Into a 2 ml microcentrifuge tube, pipet 20 µl (400 µg) of proteinase K solution in 10 mM Tris-HCl, 1 mM Calcium Chloride, 50% glycerol, pH 7.5.

Table 1. DNA Yield From Whole Blood
Using MagaCell™ Or QIAGEN QIAamp DNA Mini Kit

	PCR Quantitation (μg)		A_{260} Quantitation (μg)	
Sample ID	MagaCell™	QIAamp	MegaCell™	QIAamp
A	12.13	12.61	10.57	6.01
B	6.13	5.91	8.75	4.89
C	4.84	7.11	8.23	5.24
D	6.11	5.97	8.28	4.14
E	3.84*	9.58	7.10*	6.95

*Eluted only once.

Table 2. DNA Yield From Whole Blood
Using MagaCell™ Or QIAGEN QIAamp DNA Mini Kit

	MegaCell™	QIAamp
Sample ID	DNA Copies (Total)	DNA Copies (Total)
A	$1.17 \times 10^{6*}$	2.91×10^{6}
B	3.69×10^{6}	3.84×10^{6}
C	3.71×10^{6}	1.80×10^{6}
D	4.64×10^{6}	2.16×10^{6}
E	6.14×10^{6}	1.82×10^{6}

*Eluted only once.

The method described herein is simple, fast, economical, and produces high-yield purified DNA, comparable to or better than those produced by using a leading supplier of the DNA isolation product (Qiagen, Valencia, CA).

EXAMPLE 9

DNA Isolation From Cultured Cells Using
Magnetizable Cellulose and a Modified Wash Buffer

DNA from cultured cells (maximum $2.5x10^7$ cells) suspended in 200 µl PBS (Phosphate Buffered Saline) was isolated and analyzed as in Example 5.

EXAMPLE 10

DNA Isolation From Plant Tissue Using Magnetizable Cellulose

DNA from Arabidopsis plant leaves (obtained from Department of Plant Biology, University of Davis, Davis, CA) was released using Proteinase K (PK) and a Lysis Buffer. The DNA was then bound to MagaCell Particles in presence of the Binding Buffer. The DNA bound to MagaCell Particles was then separated and washed from other contaminants. The DNA was eluted from the particles. The following procedure was used:

1. Place 25-100 mg of a well-ground plant tissue at the bottom of a 2 ml microcentrifuge tube.
2. Add 200 µl of the Lysis Buffer A (Buffer ATL, Qiagen, Valencia, CA, Catalog Number: 19076), followed by 20 µl of the PK Solution. Mix gently by pulse vortexing. Note: If RNA-free DNA preparation is required, add 10 µl of a 40 mg/ml RNase A stock solution before addition of the Plant Lysis Buffer.
3. Incubate at 65°C for 15 minutes.
4. Remove the tube from 65°C.
5. Centrifuge at maximum speed in a microcentrifuge for 5 min.
6. Gently transfer the supernate into a clean 2 ml microcentrifuge tube.
7. Add 500 µl of the Binding Buffer (10% PEG 8000 MW, 1.25 M NaCl), followed by 20 µl of the well-mixed (particles are uniformly suspended) MagaCell Particles.
8. Mix the tube gently and incubate for 10 min at room temperature, while mixing (using an end-over-end rotator or manual mixing).

1. Place ~5 mg of a fish fin tissue at the bottom of a 2 ml microcentrifuge tube.
2. Add 200 µl of the Lysis Buffer A (Buffer ATL, Qiagen, Valencia, CA, Catalog Number: 19076), followed by 20 µl of the PK Solution. Mix gently by pulse vortexing. Note: If RNA-free DNA preparation is required, add 10 µl of a 40 mg/ml RNase A stock solution before addition of Lysis Buffer A.
3. Incubate at 56°C with occasional mixing for 1 hour.
4. Remove the tube from 56°C.
5. Add 200 µl of the Lysis Buffer B (50 mM Tris-HCl, 50 mM EDTA, 6 M Guanidine-HCl, 6 M Urea, 10 mM Calcium Chloride, 10% Tween-20, pH 6.3).
6. Incubate at 70°C for 10 minutes, then remove the tube from 70°C.
7. Add 500 µl of the Binding Buffer (10% PEG 8000 MW, 1.25 M NaCl) followed by 20 µl of the well-mixed (particles are uniformly suspended) MagaCell Particles.
8. Mix the tube gently and incubate for 10 min at room temperature, while mixing (using an end-over-end rotator or manual mixing).
9. Sediment the Magacell bound DNA particles using a magnetic rack. Aspirate the supernate and wash particles as described in Step 10.
10. Add 1 ml Wash Buffer (10% PEG 8000 MW, 0.5 M NaCl) to the tube from Step 9. Mix well, sediment the particles on the magnetic rack and aspirate the supernate.
11. Repeat the wash once more by following Step 10.
12. Add 200 µl of the Elution Buffer (10 mM Tris, pH 8.0, 1 mM EDTA) or deionized water and mix for 10 min as in Step 8.
13. Sediment the particles and carefully transfer the supernate containing the isolated DNA into a clean tube. The material is ready for further analysis. If the sample is not going to be tested on the same day, freeze at -20°C until the time of analysis.

4. Centrifuge at high speed for 10 min.
5. Carefully remove the superanate and transfer into a clean 2 ml microcentrifuge tube.
6. Add 500 µl of the Binding Buffer (10% PEG 8000 MW, 1.25 M NaCl) followed by 20 µl of the well-mixed (particles are uniformly suspended) MagaCell Particles.
7. Mix the tube gently and incubate for 10 min at room temperature, while mixing (using an end-over-end rotator or manual mixing).
8. Sediment the MagaCell bound DNA particles using a magnetic rack. Aspirate the supernate and wash particles as described in Step 9.
9. Add 1 ml Wash Buffer (10% PEG 8000 MW, 1 M NaCl) to the tube from Step 8. Mix well, sediment the particles on the magnetic rack and aspirate the supernate.
10. Repeat the wash once more by following Step 9.
11. Add 200 µl of the Elution Buffer (10 mM Tris, pH 8.0, 1 mM EDTA) or deionized water and mix for 10 min as in Step 7.
12. Sediment the particles and carefully transfer the supernate containing the isolated DNA into a clean tube. The material is ready for further analysis. If the sample is not going to be tested on the same day, freeze at -20°C until the time of analysis.

Agarose gel electrophoresis of two different plasmid DNA samples isolated from bacterial cell lysates, using the present method of invention, showed results comparable to those obtained by QIAprep Miniprep (Qiagen, Valencia, CA), the leading supplier of plasmid DNA isolation kits (Figure 2).

EXAMPLE 15

Plasmid DNA Isolation From Bacterial Cells Using Magnetizable Cellulose and a Modified Wash Buffer

Plasmid DNA (PBA8 and PBA117, obtained from Prozyme, San Leandro, CA) was released from bacterial cell culture (E.coli: XL1-Blue), isolated to high purity, and

Real Time RT-PCR quantitation of MS2 viral RNA isolated by the present method of invention is shown in Figure 3.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

d) contacting the magentizable cellulose-bound nucleic acids separated in c) with an elution buffer to release the bound nucleic acids from the magnetizable cellulose and into the elution buffer; and

e) separating the magnetizable cellulose from the elution buffer to provide nucleic acids that are substantially free of the non-nucleic acid materials.

8. The method of claim 7, wherein the separation of the magnetizable cellulose particles in step c)and e) is carried out magnetically.

9. The method of claim 8, wherein the nucleic acids bound to magnetizable cellulose particles are DNA and are washed with a wash buffer, wherein the wash buffer removes impurities bound to the magnetizable cellulose particles while leaving the DNA bound to the magnetizable cellulose particles.

10. The method of claim 9, wherein the DNA bound to the magnetizable cellulose particles is eluted with an elution buffer that releases the DNA bound to the magnetizable particles.

11. The method of claim 10, wherein the DNA released by the elution buffer is isolated.

12. The method of claim 7, wherein the polyethylene glycol has a molecular weight of 8000, and wherein the salt is sodium chloride.

13. The method of claim 12, wherein the concentration of polyethylene glycol is about 10%, and concentration of sodium chloride is between 0.25 M to 5.0 M.

14. The method of claim 7, wherein the nucleic acids and non-nucleic acid materials are obtained from a cell lysate.

15. The method of claim 14, wherein the lysate is prepared from cells of human, animal, plant, viral or bacterial origin.

16. A kit for isolation and purification of nucleic acids, comprising magnetizable cellulose and reagents at suitable concentrations for isolating nucleic acids from various sources.

derivatives, producing a second combination comprising magnetizable cellulose derivative-bound nucleic acids;

c) separating the magnetizable cellulose derivative-bound nucleic acids from the second combination;

d) contacting the magnetizable cellulose derivative-bound nucleic acids separated in c) with an elution buffer to release the bound nucleic acids from the magnetizable cellulose derivatives and into the elution buffer; and

e) separating the magnetizable cellulose derivatives from the elution buffer to provide nucleic acids that are substantially free of the non-nucleic acid materials.

25. The method of claim **24**, wherein the separation of the magnetizable cellulose derivatives in step c)and e) is carried out magnetically.

26. The method of claim **24**, wherein the nucleic acids bound to magnetizable cellulose derivatives are washed with a wash buffer, wherein the wash buffer removes impurities bound to the magnetizable cellulose derivatives while leaving the nucleic acids bound to the magnetizable cellulose derivatives.

27. The method of claim **26**, wherein the nucleic acids bound to the magnetizable cellulose derivatives are DNA and are eluted with an elution buffer, wherein the elution buffer releases the DNA bound to the magnetizable cellulose derivatives.

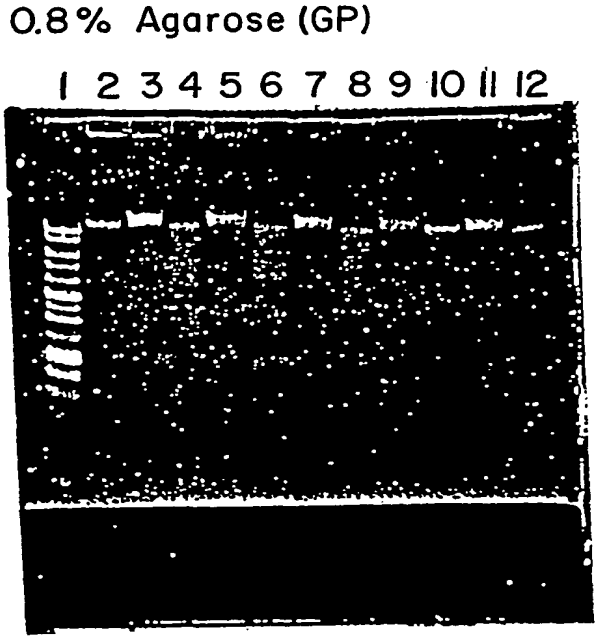
28. The method of claim **27**, wherein the DNA released by the elution buffer is isolated.

29. The method of claim **24**, wherein the polyethylene glycol has an average molecular weight of about 8000, and wherein the salt is sodium chloride.

30. The method of claim **29**, wherein the concentration of polyethylene glycol is about 10%, and the salt concentration is between 0.25 M to 5.0 M.

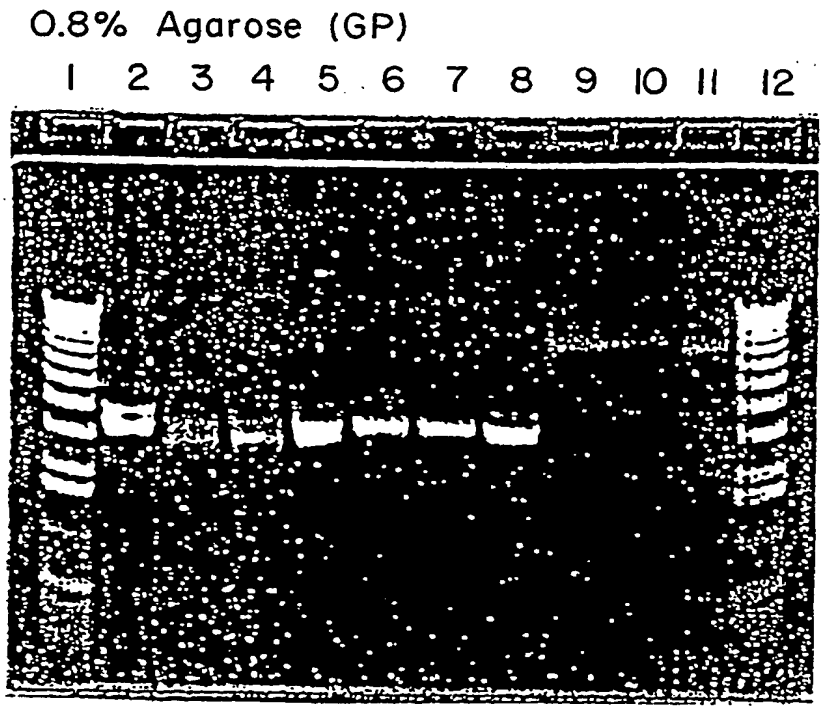
31. The method of claim **24**, wherein the nucleic acids and non-nucleic acid materials are obtained from a cell lysate.

32. The method of claim **31**, wherein the lysate is prepared from cells of human, animal, plant, viral or bacterial origin.



Lane 1: 1 Kb DNA Ladder
Lane 2: Calf thymus DNA Control
Lanes 3, 5, 7, 9, and 11: DNA isolated by MagaCell
Lanes 4, 6, 8, 10, and 12: DNA isolated by QIAamp

FIG. 1.



Lanes 1 and 12: 1 Kb DNA Ladder
Lane 2: Plasmid DNA PBA117 Control
Lane 3, 4, 6, and 7: Plasmid DNA PBA117 isolated by MagaCell
Lane 5 and 8: Plasmid DNA PBA117 isolated by QIAprep Miniprep
Lanes 9 and 10: Plasmid DNA PBA8 isolated by MagaCell
Lane 11: Plasmid DNA PBA8 isolated by QIA prep Miniprep

FIG. 2.

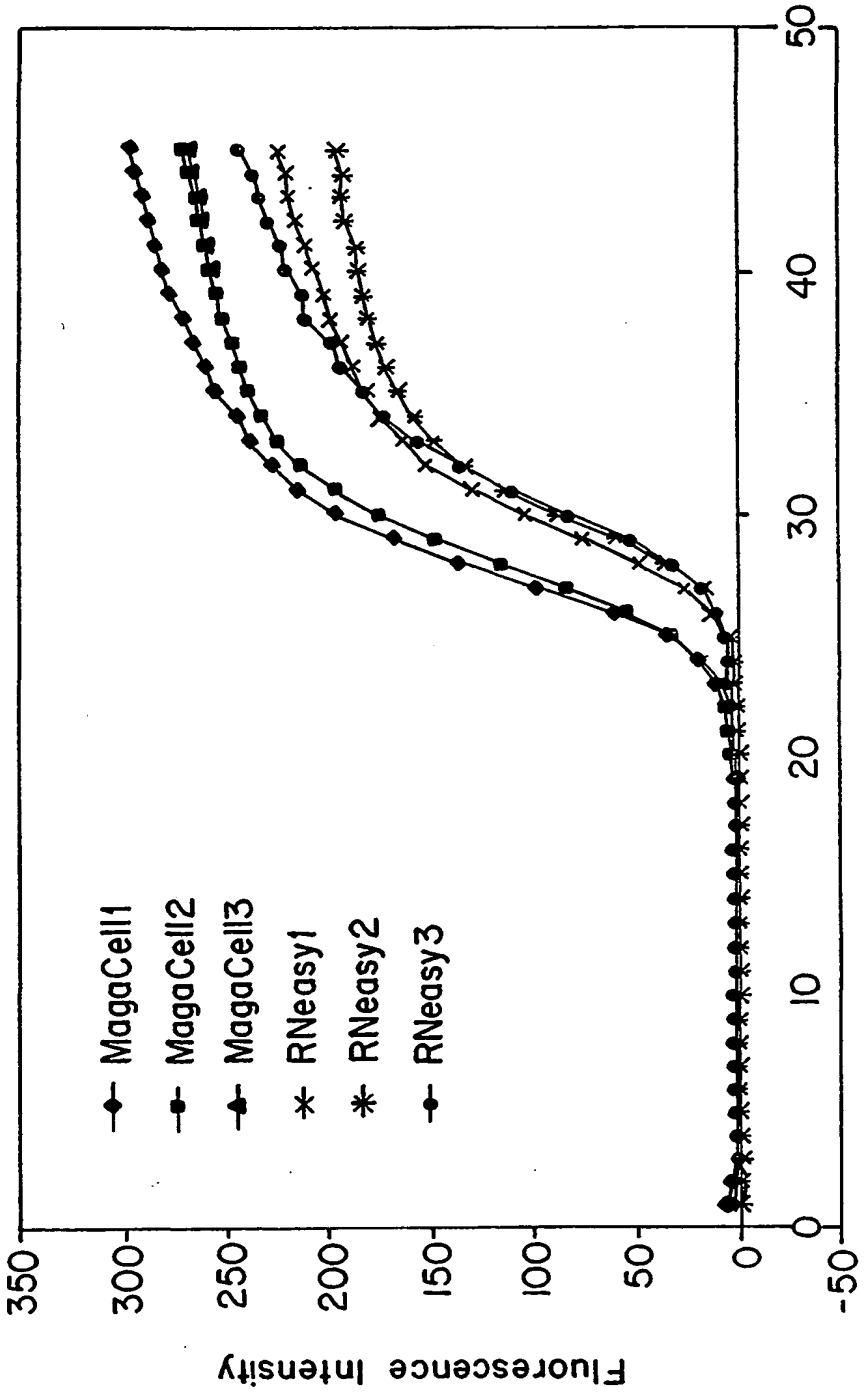

MagaCell1
MagaCell2
MagaCell3
RNeasy1
RNeasy2
RNeasy3
Fluorescence Intensity
Cycle Number
350
300
250
200
150
100
50
0
-50
0
10
20
30
40
50

FIG. 3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/31637

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 033/543, 033/546; C12N 001/08
US CL : 435/6, 7.5, 7.9, 7.92, 7.94, 270; 436/ 178, 501, 518

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 7.5, 7.9, 7.92, 7.94, 270; 436/ 178, 501, 518

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5437,983 A (WATTS et al) 1 August 1995 (1.08.1995), col.4, lines 1-5.	1-33
A	US 4,921,805 (GEBEYEHU et al) 1 May 1990 (01.05.1990), col. 5, lines 12-31.	1-33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
10 January 2002 (10.01.2002)	13 FEB 2002
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